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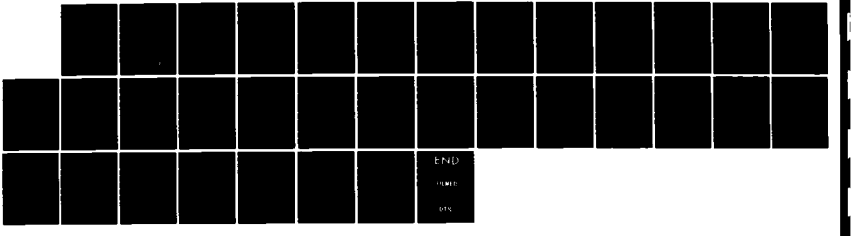

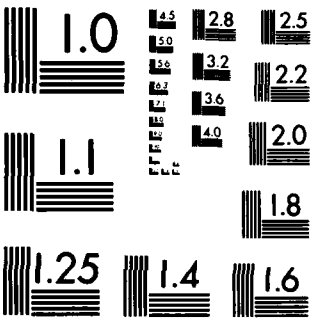

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TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUS DISEASES OF MILITARY IMPORTANCE IN EQUATORIAL ASIA

Annual Report

Dr. George F. de Witt
LTC (P) Michael G. Groves

September 1982

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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The plaquing and cloning procedures have been established and studies requiring cloning are underway. Preliminary results of characterizations indicate that the Karp and Karp-related strains are most prevalent in the endemic region. The electron microscopy study of infected endothelial cells is nearing completion; micrographs are being examined/interpreted. Cell mediated immunity studies suggest that immunity to scrub typhus can be predicted using the lymphocyte transformation assay, although this will require confirmation by

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proposed studies. The stability of the avirulence characteristic of several R. tsutsugamushi strains has been demonstrated by testing after 25 continuous passages in mice or cultured cells.

Vector transmission studies have shown that infection does not appear to influence the sex ratio of infected siblings, that mites are not infected by feeding on infected mite eggs, and the minimum attachment time for vector chiggers tested varies from $3\frac{1}{2}$ to 9 hours. Also, chromosome studies have shown that the diploid number of chromosomes in different species of vector mites is not the same. Work with the latex agglutination technique as an early diagnostic tool continues, and current work centers around testing for use with human serum specimens. The reinfection study using volunteers who had received chemoprophylaxis during our previous doxycycline trial was very successful, 7 of the 8 resisting the challenge by infected chiggers.

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SUMMARY

The ultimate goal of the research program conducted by the USAMRU-M, IMR is the elimination of scrub typhus as a military and civilian health problem. To reach this goal, the scientists at the IMR are pursuing studies on: (i) the cloning of Rickettsia tsutsugamushi to ascertain the cause of the multiple-reactivity seen on serological testing of isolates, (ii) the isolation of avirulent clones of R. tsutsugamushi in an effort to develop a living, avirulent vaccine, (iii) the infection of cultured human endothelial cells in order to examine the growth and cytopathology of the organism in cells considered to be the "target cells" in scrub typhus pathogenesis, (iv) the development of a test(s) that will predict immunity to scrub typhus by measuring the cell-mediated immune response in infected humans, (v) the testing of a mouse model for use in screening R. tsutsugamushi isolates for avirulence, (vi) the study of the disease vector to increase our knowledge of the mechanism of transmission of the organism and the role of mite genetics in this process, (vii) the development of a test(s) that will give us a more rapid means of definitively diagnosing scrub typhus, (viii) the immune response in persons infected with R. tsutsugamushi while on chemoprophylaxis, and (ix) the analysis of available epidemiological data on scrub typhus in Malaysia to further our knowledge of this disease.

The plaquing and cloning procedures have been established in this laboratory and studies requiring cloning of the organism are underway. This is a long and tedious process and it is anticipated that these studies will require several years or more for their completion. Preliminary results of our characterization study indicate that the Karp and Karp-related strains are the most prevalent in the endemic region. Isolates are continually being received and characterized to add to and update this data. The electron microscopy study of infected endothelial cells is nearing completion; electron micrographs are presently being examined/interpreted. Results of cell mediated immunity studies suggest that immunity to scrub typhus can be predicted using the lymphocyte transformation assay, although this will require confirmation by proposed studies. Known avirulent strains of R. tsutsugamushi have been shown to be stable in that they do not become virulent in mice or cultured cells after 25 continuous passages.

Transmission studies of R. tsutsugamushi by vector mites have shown that rickettsial infection does not appear to influence the sex ratio of infected siblings, that mites do not become infected by feeding on infected mite eggs, and that the minimum attachment time for vector chiggers tested varies from $3\frac{1}{2}$ to 9 hours. In addition, chromosome studies have shown that the diploid number of chromosomes in different species of vector mites is not the same. Work with the latex agglutination technique as an early diagnostic tool is continuing, with current work centered around testing of the procedure for use with human serum specimens. The reinfection study using volunteers who had received chemoprophylaxis during our previous doxycycline trial was very successful, with 7 of the 8 resisting the challenge by infected chiggers. Finally, 3 manuscripts dealing with the analysis of previously collected data on the

epidemiology of scrub typhus in Malaysia have been prepared and submitted for publication; with 2 more to be completed this year.

Most of the studies not completed have been proposed for continuation into the new grant period. The majority of the research is proceeding as expected. By the end of the grant period we anticipate that several of the projects will reach completion.

FOREWORD

The research program of the U.S. Army Medical Research Unit (USAMRU-M) at the Institute for Medical Research (IMR) consists primarily of an in-depth study of scrub typhus, a disease endemic in the Asiatic-Pacific region. This program has as its ultimate goal the elimination of this disease as a medical problem. To accomplish this, specific questions relating to the epidemiology, pathogenesis, diagnosis, treatment and prevention of the disease are being addressed in collaborative studies involving scientists in the Malaysian Ministry of Health and the USAMRU-M, a division of the IMR.

It is important to note that this annual report covers only the first eight months of the one year period. Several of the project reports are not as detailed as they would be upon completion of the grant year, due to the number of months required to get the work to the point where results are being seen, collected, and/or analyzed.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, (DHEW Publication No. (NIH) 78-23, revised 1978).

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CLONING OF RICKETTSIA TSUTSUGAMUSHI AND ISOLATION OF AVIRULENT STRAINS

Statement of the Problem: The objectives of this project are: (i) to determine if certain isolates of Rickettsia tsutsugamushi are composed of single strains or are mixtures of two or more strains, (ii) to isolate clones of R. tsutsugamushi that are avirulent in mouse and monkey models, and (iii) to examine the shifts in predominant antigens that occur from one generation of R. tsutsugamushi-infected Trombiculid mites to the next generation.

Background: Antigenic heterogeneity is a well established characteristic of R. tsutsugamushi strains. Many isolates from humans, rodents, and chiggers have been shown by the direct fluorescent antibody (FA) test to be multiply-reactive to as many as six strains (1-7). The determination of whether this multiple reactivity is the result of a mixture of strains or of a single strain expressing a mosaic of antigens is important to the selection of potential vaccine strains.

The predominant antigens in R. tsutsugamushi isolates from countries in the endemic region are Karp or Karp-related (2-9). The Karp-related antigens are those expressed by the TA716, TA763 and TA686 strains, two of which (TA716 and TA686) are avirulent for mice. The majority of isolates that react with the Karp-related strain antigens by direct FA appear to be mixtures of two or more strains.

Characterization of scrub typhus rickettsial antigens in infected Leptotrombidium arenicola and L. fletcheri chiggers by direct FA reveals that there are shifts in the predominant antigen(s) from one generation to the next (4). Knowledge of the reason for this phenomenon could be important to vaccine development, particularly if the shifts are due to a variance in the expression of specific antigens by a single strain of R. tsutsugamushi.

Approach to the Problem: The use of the plaquing technique (10) to clone isolates of R. tsutsugamushi will allow us to find the answer to the fundamental questions mentioned above. This technique allows us to separate out individual clones from a particular isolate, which can then be tested for reactivity to each of the eight prototype strain antigens by the direct FA test. If an isolate contains a mixture of several strains, we should be able to find clones that are reactive to only one strain each.

Cloning of isolates to satisfy our first and third objectives will be done in irradiated L-929 cells. However, for isolation of avirulent clones (objective ii), it will be necessary to establish a procedure for plaquing in a cell line suitable for vaccine work.

Results and Discussion: The plaquing and cloning procedures, using irradiated L-929 cells, have been successfully introduced into this laboratory. Unfortunately, the sole source of gamma radiation in the

Kuala Lumpur area was unavailable for a $3\frac{1}{2}$ month period (April-July), due to its being relocated. Work is now continuing on the cloning of local R. tsutsugamushi isolates. The first clones should be available late this year.

The MRC-5 diploid cell line has been acquired by the USAMRU-M from the American Type Culture Collection, is growing well, and stocks have been frozen away for back-up use. This cell line is acceptable for use in vaccine development and production. We are presently working out the parameters necessary for the plaquing of R. tsutsugamushi in monolayers of these cells. We anticipate this will require 8-10 months.

The plaquing of isolates from successive generations of chiggers is of a lower priority and has been delayed.

Conclusions and Recommendations: This multiple objective project is so large and complex that we are proposing that it be split into individual projects during the 1983/1984 period. Due to the high priority we have placed on completing the first two objectives, and the labor intensive characteristic of each, we have deleted the third objective.

ANTIGENIC ANALYSIS OF RICKETTSIA TSUTSUGAMUSHI STRAINS FROM ENDEMIC AREAS

Statement of the Problem: The objective of this project is to determine the geographic distribution of strains of Rickettsia tsutsugamushi within the endemic region.

Background: Information on the prevalence and distribution of R. tsutsugamushi strains is an essential part of a program to develop an effective scrub typhus vaccine. Thus far, research in this laboratory has shown that five of the eight prototype strains are predominant in isolates from Peninsular Malaysia, Thailand, Taiwan, the Philippines, Hong Kong, Australia and the islands of the Northern New Hebrides and Santa Cruz groups (2-9). While these areas represent a large segment of the endemic region, isolates from some of the countries on the periphery have not yet been obtained and characterized.

Approach to the Problem: The USAMRU-M has been collecting samples from various countries in the Asiatic-Pacific area for several years. These are being characterized using the direct fluorescent antibody (FA) test and a distribution pattern for each antigenic type is being developed. This will guide us in determining which strains must be included in a vaccine in order for it to be effective.

A recent technological advancement has allowed for the production of hybrid cell lines (hybridomas) which make monoclonal antibodies. These antibodies react with a single antigenic determinant on an organism, thus making it possible to discriminate between cross-reacting strains by isolating hybridomas that secrete antibody specific for an antigen exclusive to each strain of the organism. A battery of these monoclonal antibodies, used to prepare conjugates for direct FA, would allow for a very detailed antigenic analysis of new and existing R. tsutsugamushi isolates.

Results and Discussion: Isolates of R. tsutsugamushi from human, animal, and chigger sources have been obtained from Malaysia, Australia, Thailand, the Philippine Islands, Melanesia, the Pescadores Islands (Taiwan), and China. These have been partially characterized, using the direct FA test. The isolates from China were only recently received and are in the process of being propagated and characterized. Isolates from Japan, West Pakistan, and other countries are being actively sought in an effort to produce a more complete description of the distribution and prevalence of strains.

The hybridoma technology (11) has been introduced into the laboratory and several hybridomas producing antibodies specific for the TA716 strain have been isolated. However, upon recovery of these hybridomas from the frozen state (liquid N_2), we found that the antibody titer of ascitic fluid induced by the hybrid cells was substantially lower than prior to freezing (1:6,400 compared with 1:200). This was unexpected yet not without precedence. We are recloning these lines to determine if this was due to a non-producer which has overgrown the original line.

In attempting to develop hybridomas specific for other strains of R. tsutsugamushi, we have been experiencing very low yields of antibody producers, which on cloning, are being lost. The low yield appears to be due to the fact that antibody titers in the mice, used for obtaining spleen cells for the fusions, are low (1:50-1:200). It has been suggested that there is a direct relation between the yield of antibody producing hybrids and the antibody response of the donor animal. Modifications are being made to the mouse immunization procedure in order to stimulate a better response.

Conclusions and Recommendations: We have developed a good perspective of the prevalence of the different strains in most of the scrub typhus endemic region. During the coming year, we will obtain and characterize isolates from those areas for which we have little or no reliable information. We have proposed the continuation of this vital project in the 1983/1984 grant proposal.

In regard to the hybridomas, we should have several new hybridomas available before the end of this grant period (31 Dec 82).

RICKETTSIA TSUTSUGAMUSHI INFECTION OF HUMAN ENDOTHELIAL CELLS IN VITRO

Statement of the Problem: The objective of this project is to examine the growth characteristics and pathologic effects of Rickettsia tsutsugamushi in cultured human endothelial cells.

Background: R. tsutsugamushi has been grown in primary cells and established cell lines, but a comparison of these in vitro studies to the in vivo pathogenesis of experimental infection or clinical disease is hampered by several factors. Established cell lines represent cells that have "dedifferentiated" both structurally and functionally from their tissue of origin. In addition, previous work has frequently been done using irradiation or chemical treatment of the cells to inhibit their multiplication. Endothelial cells, however, maintain their differentiated structural and functional attributes when cultured in vitro (12-14). Since the endothelial cell appears to be a key "target cell" in the scrub typhus disease process (15-19), this model system is being explored as an ideal system for controlled studies of the growth and cytopathology of R. tsutsugamushi.

Approach to the Problem: Cultured human endothelial cells, both R. tsutsugamushi infected and uninfected, are being examined by light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). In addition, the growth rate of the rickettsiae is being determined by titration of infected cell samples (taken at specific time intervals) in mice. These results will be compared with those from concurrent experiments using an established cell line.

Results and Discussion: The procedure for the isolation and subculturing of endothelial cells obtained from human umbilical cords (20) has been successfully introduced into this laboratory. R. tsutsugamushi-infected and uninfected endothelial and L-929 cells have been examined by SEM. The electron micrographs are presently being examined and will be compared with the TEM photos. Specimens are being prepared for ultra-thin sectioning, staining and subsequent examination by TEM. In addition, both infected and uninfected cells will be stained with hematoxylin and eosin, a standard pathologic stain, and will be examined by light microscopy. Pathologic examination of the cells will be done in collaboration with Dr. Michael Elwell, a veterinary pathologist located at the U.S. Army Laboratory (AFRIMS) in Bangkok, Thailand.

Titrations of rickettsiae in endothelial cells on days 0, 4, 8, 12, and 16 post-infection are presently being done.

Conclusions and Recommendations: This project is progressing nicely. Due to technical problems with the TEM, we have been unable to proceed as rapidly with that portion as we have with the SEM.

We have proposed that this project be continued into the next grant period, so that we can complete it and also attempt to plaque R. tsutsugamushi in endothelial cell monolayers.

STUDIES IN CELL-MEDIATED IMMUNITY

Statement of the Problem: The objectives of these studies are: (i) to develop one or more assays to measure the cell-mediated immune response after infection with Rickettsia tsutsugamushi; (ii) to study the onset and longevity of these responses in mice, monkeys, and humans; and (iii) to determine the relevance of the assays in predicting immunity to reinfection with homologous or heterologous strains of R. tsutsugamushi.

Background: Cell-mediated immunity (CMI) plays an important role in the protection of animals and man from intracellular infections (21,22). This has been substantiated in R. tsutsugamushi infections using the mouse model. Mice are protected from heterologous strain infections by the transfer of T, but not B, lymphocytes from immune mice (23,24).

In vitro correlates of CMI have been shown for many classes of rickettsiae: Coxiella burnetii - macrophage migration inhibition (25) and lymphocyte transformation (LT) (26,27); R. typhi - LT (28); R. tsutsugamushi - suppression of rickettsial growth in mouse peritoneal cells incubated with supernatants (containing lymphokines) from immune spleen cell cultures (29).

When immune lymphocytes are incubated with antigen, non-immunoglobulin substances (which mediate cellular responses) are released into the media. One of these substances is macrophage migration inhibition factor (MIF) (30). The indirect MIF assay used in our studies compares the migration of macrophages from an agarose micro-droplet when supernatants of test or control cultures are added (31).

Antigen: We have continued to use membrane and soluble fractions of French pressure cell treated, tissue culture grown R. tsutsugamushi (32).

Mouse Studies:

Approach -- Optimal Karp and Gilliam strain antigen concentrations for differentiating immune and normal mice in LT and MIF assays were determined for BALB/c and C3H/He mouse strains. Gilliam antigen in C3H/He mice was found to be a poor discriminator of prior infection. Antigen concentrations for LT:

Mouse Strain	Infection Strain (10^3 MID_{50} S.C.)	Membrane Antigen conc. (μg/ml)	
		Karp	Gilliam
BALB/c	Karp	50	350
BALB/c	Gilliam	50	350
C3H/He	Karp	800	350
C3H/He	Gilliam	800	350

Soluble antigens (50 μg/ml) are used in the MIF assay.

To investigate the onset, longevity and correlates of scrub typhus immunity, a large group of BALB/c and C3H/He mice were infected subcutaneously with the Karp or Gilliam strain. At selected intervals, five mice from each of the four groups were tested for LT (32,33), MIF (31,32), and indirect fluorescent antibody (IFA)(34). At the same time, five mice from each group were tested for delayed-type hypersensitivity using the footpad swelling assay (35) with killed whole rickettsiae as antigen; several mice from each group were challenged with a "lethal" dose of rickettsiae to determine their immunity:

Mouse Strain	Infecting Strain	Challenge Strain
BALB/c	Karp	Karp
BALB/c	Gilliam	Karp
C3H/He	Karp	Karp, Gilliam
C3H/He	Gilliam	Karp, Gilliam

Results and Discussion: Although it was intended that each mouse be subcutaneously infected with 10^3 MID_{50}, the dilution used resulted in an inoculation of only $10^{1.3}$ MID_{50} Gilliam and $10^{0.8}$ MID_{50} Karp (titered intraperitoneally in ICR mice). IFA antibody was detected for the first time 21 days after infection. Peak titers (up to 1:400) occurred 35-56 days after infection. Only half of the Gilliam-inoculated C3H/He mice, and none of the Gilliam-inoculated BALB/c mice, developed antibody. This reflects the low and perhaps patchy inoculum that the mice received.

Back-challenge: Karp-infected mice were solidly immune to back-challenge of Karp (in BALB/c) and Karp or Gilliam (in C3H/He) from day 14, the first day tested. Gilliam-infected C3H/He were partially immune to Karp or Gilliam challenge from day 21 but never achieved more than 60% protection (three mice out of five). No Gilliam inoculated BALB/c mice survived Karp challenge. This result is undoubtedly related to the low titer of the inoculum. The same level of immunity has persisted for 112 days post infection.

Lymphocyte Transformation (LT): Mitogen responses (to PHA) were suppressed in Karp-infected BALB/c and C3H/He mice from day 11 or 14 to day 28. Stimulation indices to homologous and/or heterologous antigens were significant for most Karp-infected mice on day 7, and then were suppressed until days 28 or 35. Responses are still significant at 112 days post-infection.

Migration Inhibition Factor (MIF): The majority of supernatants derived from Karp-infected mice tested from days 7 - 28 induced migration inhibition. At the time of peak infection (days 14-21) some supernatants from cells incubated without antigen contained significant MIF activity. This activity was not enhanced in cultures of cells with antigen, and the resultant migration index (= migration distance with supernatant from antigen + cells / migration distance with supernatant from media + cells)

was greater than 0.80, the arbitrary cut-off point.

Delayed-type hypersensitivity (DTH): The antigen concentration (1.0 mg/ml) for this assay was derived using mice previously infected with 10^3 MID_{50}. None of the mice from the present study gave positive responses.

Conclusions and Recommendations: Suppression of LT to unrelated antigens after rickettsial infection has already been reported for spotted fever group rickettsiae (36). Specific LT in the mouse persists after recovery from infection with R. tsutsugamushi, and thus-far correlates with back-challenge survival. MIF production was shown in our study to occur only briefly (days 7-28 after inoculation).

The experiment is being repeated with a higher titered inoculum.

Human Studies:

Approach -- The aim of these studies is to determine the CMI responses of human lymphocytes incubated with rickettsial antigens in order to develop a test which is capable of predicting immunity to reinfection. Members of the staff of USAMRU-M have been used as donors. Their past histories include clinical scrub typhus (37), exposure to R. tsutsugamushi while taking doxycycline prophylaxis (37), or no history of scrub typhus exposure.

Results and Discussion: LT responses to the Karp, Gilliam and Kato antigens at concentrations of between five and 400 µg/ml were determined for donors with one of the three possible past histories. Concentrations of 100 and 400 µg/ml were chosen for routine use, the higher concentration being necessary to obtain a response in some subjects with previous suppressed infections. Lymphocytes from normal volunteers never responded to rickettsial antigens, even at 400 µg/ml.

Lymphocytes were isolated every six days from the eight volunteers participating in the reinfection study who had successfully taken doxycycline prophylaxis in a study 21 months earlier (37). The results of the reinfection study appear elsewhere in this report. The only two volunteers who did not have positive LT responses to rickettsial antigens before their reinfection were the same two volunteers who subsequently had significant clinical disease. One was treated for scrub typhus; the other did not fulfil the criteria established for a diagnosis of scrub typhus, and after a week of low grade fever ($\leq$36.4C) he recovered. LT in the other subjects was suppressed at some period between days 6 and 18 after challenge. Peak LT was seen at day 60. MIF assays are not complete at this time of writing but our initial impression is that the challenge infection did not cause a rise in MIF-producing lymphocytes, as measured in our assay.

Conclusions and Recommendations: A larger scale study of the in vitro correlates of scrub typhus immunity is planned for 1983 using a rural Malaysian population which has heavy exposure to scrub typhus. The results of the small, laboratory-based study suggest that a positive LT

response is predictive of immunity. A negative LT response must be interpreted in light of the antigen concentration used in the assay, but the absence of LT at 400 μg/ml was associated with more evidence of disease after challenge.

Monkey Studies:

Approach -- The onset of CMI responses in monkeys having their first exposure to R. tsutsugamushi, and the correlation of baseline responses in monkeys previously infected with R. tsutsugamushi to their clinical response after reinfection, is being studied.

Results and Discussion: Baseline LT and MIF assays have been performed, and the monkeys will be infected on 9 Oct 82.

Conclusions and Recommendations: These must await the end of the study (December 1982).

STUDIES ON THE AVIRULENCE OF RICKETTSIA TSUTSUGAMUSHI IN THE MOUSE MODEL

Statement of the Problem: The objectives of this project are twofold: (i) to devise a model mouse system for use in screening Rickettsia tsutsugamushi clones for avirulence and the ability to induce cross-protection against other cloned strains, and (ii) to test the stability of several avirulent strains of R. tsutsugamushi.

Background: Cross-protection studies in monkeys have shown that Karp-related strains of R. tsutsugamushi, avirulent for both monkeys and mice, protect in many instances as well or better than virulent strains (38). We believe this indicates that a living, avirulent scrub typhus vaccine is feasible. To be acceptable, however, an avirulent vaccine must be stable with regards to virulence.

One of the difficulties in developing an avirulent vaccine is the selection of candidate strains of R. tsutsugamushi. Following the isolation, cloning, and antigenic characterization, each clone must be tested for virulence. Those found to be avirulent must be further tested for the ability to induce cross-protection against specific heterologous strains.

Currently, there is no animal system that can be used to screen potential avirulent clones of R. tsutsugamushi as candidates for use in a vaccine to be given to humans.

Approach to the Problem: Prototype R. tsutsugamushi strains, known to be of reduced virulence for mice, will be used in developing the model. These strains will be injected subcutaneously into genetically susceptible C3H/He mice (39). Twenty-one days post-infection, these mice will be sacrificed and dilutions of their spleen cells will be prepared and injected into recipient C3H/He mice (24). Recipient animals will be challenged 24 hours after cell transfer with 10^3 MLD_{50} of either the Karp or Gilliam strain, and the results compared to a homologous system, i.e. Gilliam challenge of mice receiving Gilliam immune spleen cells or Karp challenge of mice receiving Karp immune spleen cells. As a vaccination control, a portion of each group of donor mice will be challenged on day 22 with 10^3 MLD_{50} of the Karp or Gilliam strain.

One of the principal objectives of this project is to establish a rational method for selecting potential vaccine strains. By inoculating various dilutions of spleens cells into recipient mice, we propose to quantify the protection induced by avirulent strains against virulent infections.

Even though the virulence/avirulence characteristics of R. tsutsugamushi strains appear to be extremely stable, documentation of this is important if we are to successfully pursue development of an avirulent vaccine. Therefore, serial passages of the avirulent TA678, TA686, and TA716 strains of R. tsutsugamushi were made in mice and in Vero cell monolayers. Mouse passages were done by blind passage of

spleens harvested 7 days post-infection. Vero cell monolayers were harvested after 7 days and new monolayers infected with the passage material. After every 5 passes the rickettsiae were titrated in mice to test for virulence.

Results and Discussion: Testing for stability of avirulence has been completed. The three avirulent strains of R. tsutsugamushi were each passed 25 times in ICR mice and, in a separate series, 25 passes in Vero cell monolayers. Testing for virulence was done, after every five passes, by the intraperitoneal injection of ICR mice and subsequent challenge (after 28 days) with the virulent Karp strain. A preliminary analysis of the data shows that the three strains used did not regain even partial virulence after continuous passage. Due to delays in the production of C3H/He mice and the increased mouse requirements for products of a higher priority, it was necessary to delay the mouse model portion of this project.

Conclusions and Recommendations: We anticipate that this project will be completed within the next few months.

Statement of the Problem: The objectives of this project are: (i) to examine the transmission of Rickettsia tsutsugamushi by Trombiculid mites with specific reference to the genetics of transovarial transmission and the influence of infection on the sex ratio, (ii) to develop an electrophoretic technique for use in differentiating the sex and species of mites, as well as their infection status, and (iii) to perform chromosome studies on mites to see if there is a detectable karyotypic difference between infected and uninfected mites and between mite species and sexes.

Background: Uninfected chiggers are capable of acquiring R. tsutsugamushi by feeding on infected mice (40). This infection is subsequently passed through the various developmental stages to the adult mite, but is not transmitted to succeeding generations. Using our infected and uninfected Leptotrombidium arenicola and L. fletcheri colony chiggers, previous studies of several successive generations have demonstrated very high filial and transovarial infection rates approaching 100% (41,42). In addition, these studies have also shown that the progeny of infected mites are almost exclusively female. Field studies have shown that multiple infections can occur in chiggers (3,4,6-8). There are several important questions regarding chigger transmission of rickettsiae: (i) how do chiggers become multiply-infected? (ii) does the rickettsial infection influence the sex ratio in the infected mites? (iii) can R. tsutsugamushi be transmitted by infected male mites or by cannibalism of infected mite eggs by uninfected mites? and (iv) what is the minimum attachment time required for an infected chigger to transmit scrub typhus?

Electrophoresis has become an important method for studying the genetic biology of a number of species. Isoenzyme studies in mosquitoes have focused on species relationships, formal genetics, surveys of natural populations for genetic variability, and reproductive biology and behaviour. The chigger colonies maintained by the USAMRU-M enable us to initiate similar studies on mites.

Most arthropod cytogenetic studies have been done with mosquitoes. Chromosome data are available on mite species in several families of the suborder Prostigmata; however, none of the species within the family Trombiculidae, which contains the vectors of scrub typhus, have been studied. Important questions to be answered are: (i) do any cytotaxonomic differences exist among these related species? and (ii) do any genetic mechanisms, such as sex determination, contribute to the various mite dynamics?

Approach to the Problem: The infected mites in our L. arenicola colony carry the Karp, TA686, TA716, TA763, and Kato strains, but not Gilliam (4). These and uninfected L. arenicola chiggers will be fed on mice experimentally-infected with the Gilliam strain. Their offspring will be followed for several successive generations to determine if the Gilliam

strain can be acquired and subsequently transmitted both transtadially and transovarially.

In a separate set of experiments, four lines each of uninfected and infected L. arenicola and L. fletcheri will be identified and followed through all stadia. The number of dead and their sex will be recorded for each stage. The sex determination can be done morphologically in adults and by dissection and identification of reproductive organs in the nymphophanes, nymphs, and teliophanes. Unfortunately, the sex cannot be differentiated in the larval stage. These results will be analyzed to examine the influence of infection on the sex ratio.

Large numbers of mites in our infected colonies will be allowed to mature to the adult stage and a careful search made for male mites. If one or more are found, they will be mated with uninfected females. Subsequent generations will be followed to determine the infection rate, transmission rate, and sex ratio of the progeny. In addition, nymphs and adults from uninfected mite colonies will be fed infected mite eggs and subsequent generations will be examined for possible infection by R. tsutsugamushi.

To determine the minimum attachment time, large numbers of chiggers will be fed on mice and at predetermined intervals, a portion of the chiggers will be removed from the mice. Mice will then be processed for rickettsial isolation by standard procedures. This will be done using both L. arenicola and L. fletcheri chiggers.

The electrophoretic separation of isoenzymes using starch gel electrophoresis will be attempted using newly-emerged adult L. arenicola and L. fletcheri. Infected and uninfected female and uninfected male mites will be used. If available, infected male mites will also be used. We hope to be able to differentiate sex, species, and/or infection status using isoenzyme characterizations.

Finally, using well-established karyotyping techniques, we will attempt to find species and/or sex differences among infected and uninfected L. arenicola and L. fletcheri mites.

Results and Discussion: Evidence accumulated during the present grant period suggests the following: (i) rickettsial infection does not appear to influence the sex ratio in infected mites, (ii) mites appear to be unable to acquire an R. tsutsugamushi infection by feeding on infected mite eggs, (iii) infected males are not produced by infected female mites, and (iv) the minimum attachment times required for L. fletcheri and L. arenicola chiggers to transmit scrub typhus are $3\frac{1}{2}$ and 9 hours, respectively.

In addition, one of five infected L. arenicola lines tested was able to become infected with and transovarially transmit an additional strain of R. tsutsugamushi, the Gilliam strain. However, that strain was not recovered beyond the first generation.

The isoenzyme patterns of two enzymes, phosphohexoisomerase and phosphoglucomutase, were compared for each of the laboratory mite colonies

available, as well as some field-collected L. fletcheri. The results are currently being analyzed.

Cytogenetic studies have been done as described above. We have found that the mitotic chromosomes of L. deliense and L. fletcheri show 2n = 14, while those of L. arenicola indicate 2n = 28.

Conclusions and Recommendations: In view of the limited success of our attempt to infect L. arenicola chiggers with the Gilliam strain and establish transovarial transmission, we feel that a repeat experiment is warranted. This has been programmed into our 1983/1984 grant request. In addition, the inability of the mites to become infected by feeding on infected eggs brings up the possibility that this might be due to a gut barrier. We have also requested funding of this study in the 1983/1984 grant proposal.

Additional studies proposed deal with the electrophoretic separation of isoenzymes and the differentiation of karyotypes. Our results to date justify a continuance of these projects.

EARLY DIAGNOSIS OF RICKETTSIA TSUTSUGAMUSHI INFECTIONS

Statement of the Problem: The objective of this project is to develop one or more laboratory tests for obtaining a more rapid definitive diagnosis of scrub typhus in humans.

Background: The conventional means of diagnosing scrub typhus relies mainly on clinical signs and symptoms, with laboratory confirmation by serological methods 2-3 weeks after onset of illness. Relevant epidemiological data are often also useful. Our experience, however, has shown that the recognition of clinical disease is often difficult in highly endemic areas, because classical signs of scrub typhus (like eschar and rash) are seldom observed (43). Fever and headache are of little help diagnostically since they are also observed in many other infectious diseases. Isolation of Rickettsia tsutsugamushi using mouse inoculation often requires two or more months before a definitive diagnosis is made. More sensitive and specific serological methods or a more rapid means of isolating and identifying the organisms are needed.

Appraoch to the Problem: Several approaches to early diagnosis will be used. One procedure, latex agglutination, focuses on antigen detection; two others, radioimmunoassay and reverse solid phase ELISA (enzyme linked immunosorbant assay) detect antibodies; and the last, surface antigen detection, will look for markers found on the surface of infected cells.

Results and Discussion: Preliminary investigations have shown that agglutination does occur between R. tsutsugamushi and latex particles coated with scrub typhus antibody. These investigations have dealt only with the specificity and sensitivity of the procedure, and results have been promising.

Preliminary work on the radioimmunoassay and reverse solid phase ELISA (IgM capture) procedures was not encouraging. Priorities were redirected to the more promising techniques, latex agglutination and surface antigen detection.

In earlier work we had an indication of the possible presence of rickettsial-associated antigen on the surface of R. tsutsugamushi-infected monocytes and lymphocytes. There is a precedent for this, as virus infected cells frequently express viral antigens on their cell membranes (44,45).

Antibody against reticuloendothelial cells (RBC), from C57Bl/6 mice infected with the Gilliam strain of R. tsutsugamushi, was prepared in CBA/Ca mice. This antibody, after absorption with normal C57Bl/6 REC, was conjugated with fluorescein isothiocyanate (FITC) and tested against spleen cells of normal and acutely-infected BALB/c mice. The results indicated that the FITC-conjugated anti-REC antibody bound only to the spleen cells from the infected mice. However, the titer of this antibody was low and dilution above 1:2 was ineffective in the assay.

Conclusions and Recommendations: We are continuing to develop the latex agglutination test. We propose to test its ability to detect antigen in sera from experimentally-infected animals and eventually, from acutely-ill, human patients. This test is one that can easily be done in the field, using pre-prepared antibody-coated, latex beads.

We are presently working on obtaining a higher-titered anti-REC antiserum for use in further testing of the surface antigen test. Mice infected with the Karp, Gilliam, and Kato strains of R. tsutsugamushi will be tested at specific intervals post-infection to determine the potential of this antibody for use as an early diagnostic tool. Human peripheral blood lymphocytes from known scrub typhus cases, as well as from uninfected control individuals, will also be used in evaluating this procedure.

REINFECTION OF VOLUNTEERS PREVIOUSLY EXPOSED TO RICKETTSIA TSUTSUGAMUSHI WHILE ON DOXYCYCLINE PROPHYLAXIS

Statement of the Problem: The objective of this study was to determine if persons infected with Rickettsia tsutsugamushi while receiving weekly doxycycline, as a scrub typhus chemoprophylactic, developed immunity to the disease.

Background: In a recently completed study (37), human volunteers receiving weekly doxycycline chemoprophylaxis and deliberately infected with R. tsutsugamushi via Leptotrombidium fletcheri chiggers were protected from acquiring scrub typhus. Although immunity lasting for two or more years is known to result from clinical scrub typhus, the immune status of persons receiving chemoprophylaxis at the time of infection is unknown.

Approach to the Problem: We proposed to reinfect the original volunteers from the previous study to examine their immune status after combined chemoprophylaxis and infection. The volunteers were reinfected using L. fletcheri chiggers from the same infected colony as in the original doxycycline study. Complete physical examinations were given prior to the challenge and volunteers were examined daily for signs and symptoms of scrub typhus. Blood samples, drawn at regular intervals, were tested for the level of rickettsemia by mouse inoculation and for antibody titer by the indirect fluorescent antibody test. Cell-mediated immunity studies were planned to determine whether or not we could accurately quantify the immune status prior to the challenge.

Results and Discussion: Eight volunteers were re-exposed to infection with R. tsutsugamushi 21 months after successfully completing the doxycycline prophylaxis trial. Seven of the eight resisted the rickettsial challenge, showing that long-lasting immunity to scrub typhus does develop during a suppressed infection.

Conclusions and Recommendations: We have submitted a proposal for the coming grant period, based on these highly encouraging results, to begin a multi-phase program that would culminate in the testing of an antibio-vaccine.

ANALYSIS OF DATA FROM MAJOR EPIDEMIOLOGICAL STUDIES

Statement of the Problem: The objective of this project is the analysis of epidemiological data collected during several years of work in central Peninsular Malaysia.

Background: In the early to mid-1970's, epidemiological studies were initiated at the Mentekab Hospital, the Bukit Mendi Health Center, and the Jengka Triangle in central Peninsular Malaysia. While these focused primarily on collecting data relating to scrub typhus, information was also obtained regarding other febrile illnesses, such as leptospirosis, typhoid fever, malaria, melioidosis, and dengue fever. This data was introduced into our computer and stored until programs could be written to accomplish the required analyses.

Approach to the Problem: Computer programs will be prepared that are each capable of extracting specific types of data, analyzing it, and presenting the results in a format that is useable by USAMRU-M scientists in the preparation of manuscripts.

Results and Discussion: During the present grant period, much of the data has been analyzed and two manuscripts have been prepared for publication, with a third presently being completed. Another program has been written and will be entered in the computer during the next two months.

Conclusions and Recommendations: We anticipate that the introduction of this latest program into our computer will allow us to prepare two additional manuscripts. The completion of these will complete this project.

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